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Development and characterization of a recombinant chicken single-chain Fv antibody detecting *Eimeria acervulina* sporozoite antigen

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Abstract

Chicken monoclonal antibody (mAb), 8C3, which is reactive with a sporozoite antigen of *Eimeria acervulina*, is a potential therapeutic agent against avian coccidiosis caused by *Eimeria* spp. However, production of large amounts of 8C3 mAb in cell culture system is labor intensive and not cost-effective. Accordingly, recombinant single chain variable fragment (ScFv) antibody was constructed by amplification of the V_H and V_L genes from chicken hybridoma, 8C3 and when expressed in *E. coli* gave 5 mg l^{-1}. The expressed protein showed antigen binding activity equivalent to that of the parental mAb. In addition, nucleotide sequence comparison of 8C3 gene to the germline chicken V_L genes suggested that the gene conversion with V_λ pseudogenes might contribute to the diversification of V_L genes in chickens.

Introduction

Avian coccidiosis, caused by intestinal parasites belonging to the genus *Eimeria*, results in a significant economic loss to the poultry industry worldwide (Lillehoj & Lillehoj 2000). Although anti-coccidial drugs have been effective, high costs and the increasing emergence of drug resistant parasites limit their use in the field (Chapman 1993). Currently, two immunological strategies have been envisioned. The first strategy involves the development of recombinant subunit vaccine which engenders invasion-blocking antibodies (Lawn & Rose 1982). The second strategy involves passive immunization using antibodies that actively block the invasion of parasites into host cells (Sasai *et al.* 1996). We have previously developed several chicken hybridomas which secrete mAbs against *Eimeria acervulina* sporozoites (Sasai *et al.* 1996). However major drawbacks with using chicken mAbs are limited amount of antibody and the genetic instability of chicken hybridomas (Nishinaka *et al.* 1996). Recently, recombinant antibody technology, originally developed for producing murine antibodies in *E. coli* (Winter *et al.* 1994), was successfully applied to produce chicken single chain variable fragment (ScFv) antibodies (Yamanaka *et al.* 1996). In this study, we describe the antigen binding characteristic of the recombinant chicken ScFv antibody and its DNA sequence comparison to the germline chicken V_L gene for the

investigation of gene conversion mechanism in chickens.

Materials and methods

Amplification and nucleotide sequence analysis of variable region gene

Total RNA was extracted using TRIzol (Life Technologies, Gaithersburg, MD) from chicken hybridoma cell line, 8C3, whose secreted mAb reacts with *Eimeria acervulina* antigen. cDNA was synthesized from 5 μg total RNA with oligo-dT primer using SuperScript II RNase H^- reverse transcriptase (Life technologies) and used to amplify V_H and V_L genes. PCR reaction was performed as follows; 1 cycle of 4 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, with a final extension step of 7 min at 72 °C. Each variable region genes were amplified using the oligonucleotides pairs; CKV_LB (V_L reverse primer): 5′-GCGCTGACTCAGCCGTCCT CG-3′ and CKV_LF (V_L forward primer): 5′-*ACC TCCACTGGGTTTACCGGAAGTAGAGCCTAG GACGGT*CAGGGTTGTCCCGGCCCCAAA-3′ for the variable region of light chain; CKV_HB (V_H reverse primer): 5′-*ACTTCCGGTAAACCCAGTG AAGGTAAAGGT*GCCGTGACGTTGGACGA GTCCGGGGGCGGC-3′ and CKV_HF (V_H forward primer): 5′-GGAGGAGACGATGACTT CGGT for the variable region of heavy chains. Complementary sequences of linker are shown by italics. The PCR products were separated on 1% agarose gel and recovered using in QIAEX II gel extraction kit (Qiagen). Purified PCR products were cloned into pGEM-T vector (Promega). Plasmid DNA was sequenced with an ABI 377 automatic sequencer using a big-dye terminator cycles sequencing ready kit (PE Applied Biosystem, USA). The sequences obtained were analyzed by comparing with germline sequences of heavy and light chains of CB strain (Reynaud *et al.* 1987, 1989).

Construction of ScFv genes

V_L-V_H gene constructs with intervening 217 linkers (Whitlow *et al.* 1994) were prepared by overlap-extension PCR (Horton *et al.* 1989) using 100 ng each of purified V_L and V_H genes, and *Taq* DNA polymerase (Promega) by 15 cycles for 1 min at

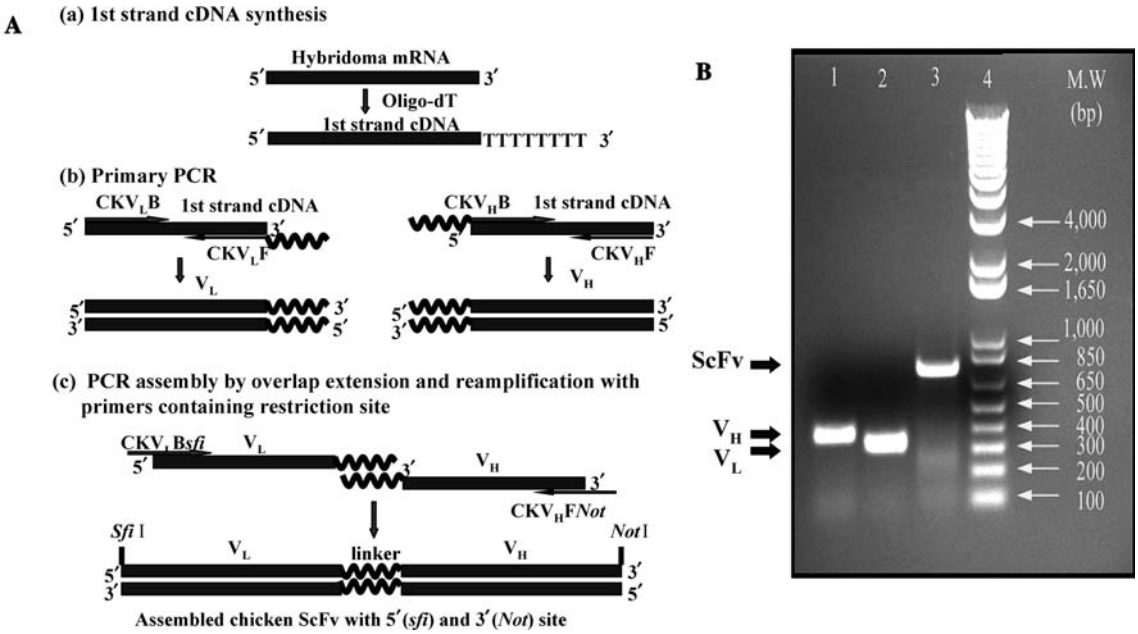

Fig. 1. Construction of ScFv, (A) PCR reactions are indicated by arrows with the primers identified next to them. Complementary linker sequences are encoded as 'add-on' sequences in 3′ ends of V_L and 5′ ends of V_H to ensure overlap extensions. (B) The PCR and overlap extension products are resolved on an agarose gel and stained with ethidium bromide: lane 1, V_H; lane 2, V_L; lane 3, ScFv; lane 4, DNA size markers.

95 °C and 4 min at 75 °C and final extension for 10 min at 72 °C (Figure 1A). The PCR products were amplified again using V_LB*Sfi*I (S*fi*I restriction site is underlined): 5′-GTCCTCGCAACTGC GGCCCAGCCGGCCATGGCCGCG-3′ and V_H F*Not*I (*Not*I restriction site is underlined): 5′-GGCCACCTTTGCGGCCGCGGAGGAGACG ATGACTTCGGT-3′. PCR was performed for 1 cycle for 4 min at 95 °C, 30 cycles for 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C and final extension for 7 min at 72 °C. Final amplified products were digested with *Sfi*I and *Not*I (Promega) and cloned into a previously characterized ScFv expression vector derived from pUC119 containing a 5′-*Pel*B leader sequence and 3′ hexahistidine tag (Kim *et al.* 1994).

Expression of 8C3 ScFv antibody gene and purification of its gene product

Plasmids containing ScFv genes were transformed into competent *E. coli* BMH71-18 (Kim *et al.* 1994). Bacteria were grown at 30 °C overnight with constant agitation (180 rpm) in TY broth (20 g tryptone, 20 g yeast extract, 10 g NaCl per liter) (Difco) containing 100 μg ampicillin ml^{-1} (Sigma) and 1% (w/v) glucose, harvested by centrifugation at $2{,}500 \times g$ for 10 min at room temperature and washed once with TY broth. The bacteria were resuspended in TY broth containing 100 μg ampicillin ml^{-1} and 1 mM isopropyl-β-D-thiogalactopyranoside and induced for 5–6 h at 25 °C with shaking at 200 rpm. To purify recombinant ScFv antibodies, bacteria were harvested by centrifugation at 4 °C and lysed in osmotic buffer (500 mM sucrose, 0.1 mM EDTA, and 200 mM Tris/HCl, pH 7.5). Cell debris was removed by centrifugation at $12{,}000 \times g$ for 30 min at 4 °C. The supernatant was dialyzed against phosphate buffered saline (PBS) and applied to Ni^+-NTA-agarose column (Peptron, Korea). Bound antibodies were recovered by 250 mM immidazole. Purified ScFv antibodies were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.125 M Tris/HCl, pH 6.8, 4% (v/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% Bromophenol Blue), heated at 94 °C for 5 min, separated on 15% SDS-PAGE using a Mighty Small II SE 260 electrophoresis apparatus (Amersham) and stained with 0.25% Coomassie Blue in 10% acetic acid/50% methanol. The expressed protein was quantitated by BCA protein assay (Pierce).

Preparation of Eimeria *antigens*

Eimeria acervulina sporozoites were prepared by incubating sporulated oocysts in 0.125% (w/v) trypsin and 1% (w/v) taurodeoxycholic acid in Hanks' balanced salt solution (all from Sigma), pH 7.6 for 10 min at 41 °C in a 5% (v/v) CO_2 incubator. Sporozoites were harvested by centrifugation and purified from cellular debris on diethylaminoethyl cellulose columns (DE52; Whatman, Maidstone, UK). Pelleted sporozoites in Dulbecco's phosphate buffered saline (PBS, Sigma) were disrupted by 6 freeze-thaw cycles, warmed to room temperature, sonicated on ice in PBS (Misonix, Farmingdale, NY) and stored at −20 °C until use.

Enzyme-linked immunosorbent assay (ELISA)

The reactivity of 8C3 ScFv to *Eimeria acervulina* antigen was determined by ELISA using 96 well plate (Nunc Maxisorp) that has been coated overnight at 4 °C with a fixed amount (1.2 μg) or various amounts of soluble *Eimeria* antigen (0.04–4 μg) in PBS. The plates were washed 3 times with PBS, pH 7.2 containing 0.1% Tween 20 (PBS-T) and blocked with 200 μl PBS containing 2% (v/v) skim milk (Gibco BRL) and 0.1% Triton X-100 (PBS-ST) for 1 h at 37 °C. After washing with PBS-T, the plates were incubated for 1 h at room temperature with various amounts (0.3–30 μg) or a fixed amount (10 μg) of a recombinant ScFv in 200 μl of PBS-ST. Following washing 3 times with PBS-T, 200 μl horseradish peroxidase-conjugated polyhistidine monoclonal antibody (Sigma) diluted 1:2,000 (v/v) in PBS-ST was added to each well and incubated for 1 h at room temperature. Finally, the plates were washed 6 times with PBS-T and developed for 30 min at room temperature with ABTS (Sigma) and H_2O_2. The plates were read at 405 nm using a microplate autoreader.

Results

Chicken V_L and V_H genes were amplified by PCR from chicken hybridoma cell line 8C3 and used to

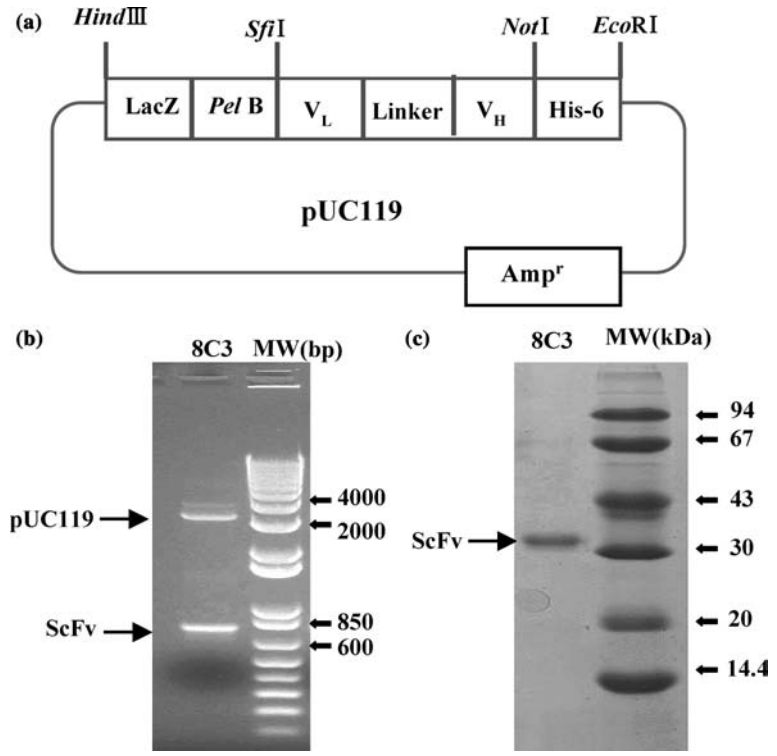

Fig. 2. (a) Schematic outline of recombinant ScFv plasmid. (b) The recombinant ScFv plasmids were digested with *Sfi*I and *Not*I and resolved on 1% agarose gel and stained with ethidium bromide. DNA size markers are shown on the right. (c) The ScFv purified from *E. coli* was resolved by SDS-PAGE under reducing conditions and stained with Coomassie Blue. The protein size markers are shown on the right.

assemble the ScFv gene construct for cloning into a prokaryotic expression vector (Figure 1). The V_L and V_H genes amplified were approximately 325 and 350 bp in length, respectively (Figure 1). Because the final ScFv gene construct included an intervening linker sequence, the final lengths of ScFvs (750 bp) were longer than expected (Figure 1) (Yamanaka *et al.* 1996). The final V_L-V_H ScFv genes (GenBank accession number AY744499) sequentially contained the following gene segments: Lac Z-*pel* B leader-V_L-217 linker-V_H-histidine6 tag (Figure 2a). The sizes of the ScFv recombinants (750 bp) were confirmed by *Sfi*I and *Not*I enzyme digestion and gel electrophoresis (Figure 2b). The recombinant ScFv construct was transformed into *E. coli*, induced with IPTG, and the recombinant ScFv antibodies purified by Ni^+-NTA affinity chromatography. Typically, 5 mg purified protein l^{-1} was obtained. As shown in Figure 2, purified recombinant 8C3 ScFv showed apparent molecular weight of 33 kDa.

Nucleotide sequences of V_L and V_H genes were determined and compared with the corresponding germline sequences from the CB strains (Figure 3a, b). Framework (FR) and complementary determining regions (CDRs) were also determined according to Kabat sequences (Kabat *et al.* 1991). In both V_L and V_H, the sequence differences between the 8C3 and germline were predominantly found in the CDRs as expected (Figure 3). For example, the CDR3 of 8C3 in the V_L genes contain the insertion of 6 nucleotides (ATTTAT) and the deletion of 3 nucleotides (AGC) (Figure 3b). Because gene conversion from V_λ pseudogenes has been hypothesized as a mechanism of generating antibody diversity in chickens, we compared 8C3 V_L sequences with 25 pseudogenes of the CB strain (Reynaud *et al.* 1987) and genes from other chicken strains (Kondo *et al.* 1993). The CDR1 and CDR2 regions of the 8C3 V_L were derived from the pseudogenes, Ψ23 and Ψ12, respectively. Similarly, the CDR3 of 8C3 V_L was derived from the pseudogene Ψ13 probably by a gene conversion process (data not shown).

The antigen binding activity of the recombinant 8C3 ScFv antibodies was determined by ELISA.

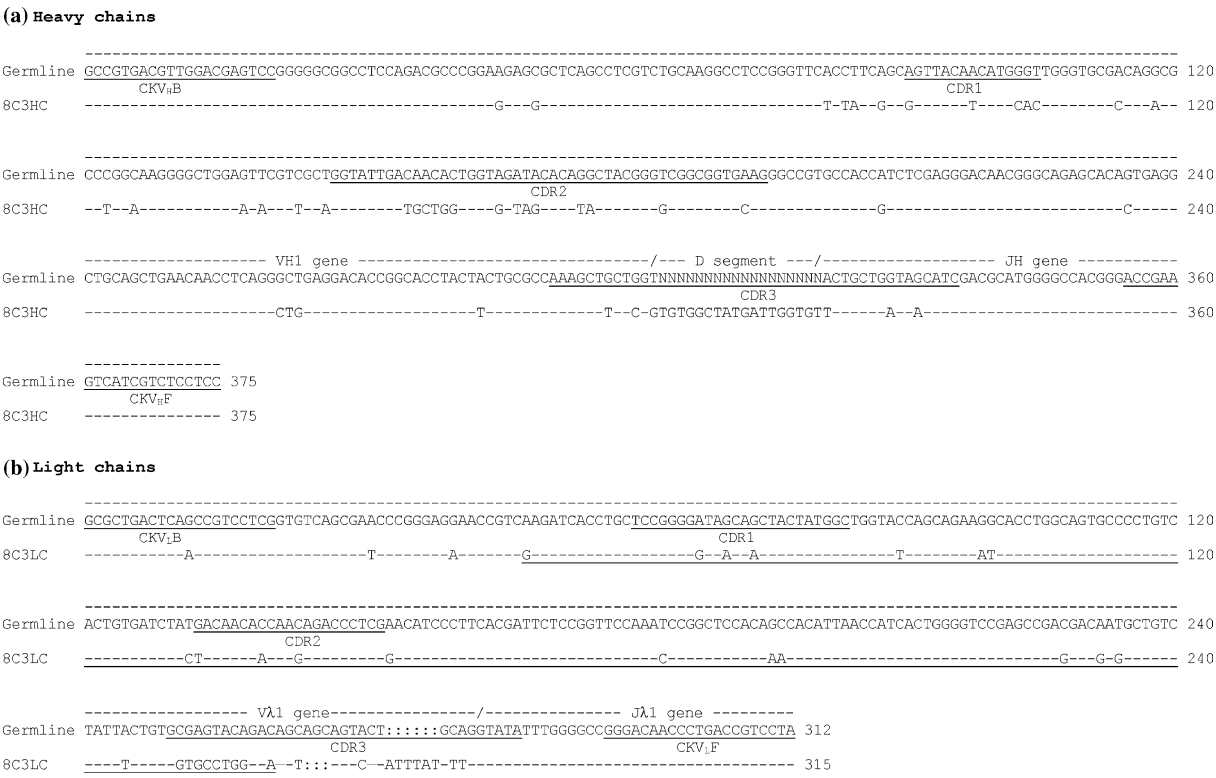

Fig. 3. Nucleotide sequences of V_H (a) and V_L (b) genes of 8C3 ScFv. Germline sequences from the CB strain are shown at the top and those from the 8C3 hybridoma cell lines below. Nucleotide identities to the germline sequences are shown by dashes (-). Nucleotide deletions are indicated by a colon (:). Complementarity-determining regions (CDRs) and PCR primers are underlined in the germline sequences. Regions to be filled with D segment in the germline heavy chain are shown by N. Nucleotides underlined in the 8C3 light chain genes indicate possible gene conversion donor pseudogene sequences.

Soluble *Eimeria* antigen was immobilized on to 96-well plates and incubated with recombinant 8C3 antibody. Increasing the amount of ScFv antibody from 0.3–30 μg resulted in the increased absorbance values when 1.2 μg antigen was used (Figure 4a). With increasing the amount of *Eimeria* antigen from 0.04–4 μg, the absorbance or antibody-antigen binding also increased (Figure 4b). These ELISA results indicated that the 8C3 ScFv antibody was reactive with soluble antigen of *E. acervulina* sporozoite in dose dependent manner.

Discussion

From chicken hybridoma cell line 8C3, we constructed ScFv recombinant antibody, expressed in *E. coli*, and tested its antigen binding activities. Like the native chicken mAbs, the recombinant 8C3 ScFv antibody showed binding activity against *Eimeria* antigen. The recombinant protein was secreted at 5 mg l^{-1} into culture medium indicating that soluble, stable and functional chicken ScFv can be produced in large volume. This suggests that the recombinant antibody technology has advantages over hybridoma technology which generally yields low quantities of antibodies (< 0.5 mg l^{-1}), generates genetically unstable hybridomas and is not amenable to produce high antibody-titer ascites (Nishinaka *et al.* 1996).

In most cases, ScFv antibody fragments have been produced in *E. coli* as insoluble inclusion bodies which are inactive unless they are solubilized and refolded (Huston *et al.* 1988). Thus, secretion of ScFv antibodies as soluble and bioactive antibodies would greatly simplify the purification procedure with high yields (Glockshuber *et al.* 1990). The order of V_H and V_L has been suggested to play a role in increasing solubility (Anand *et al.* 1991, Whitlow *et al.* 1994). The recombinant 8C3 ScFv antibody constructed as the V_L-V_H chain (L-H) exhibited better secretion compared to the antibody

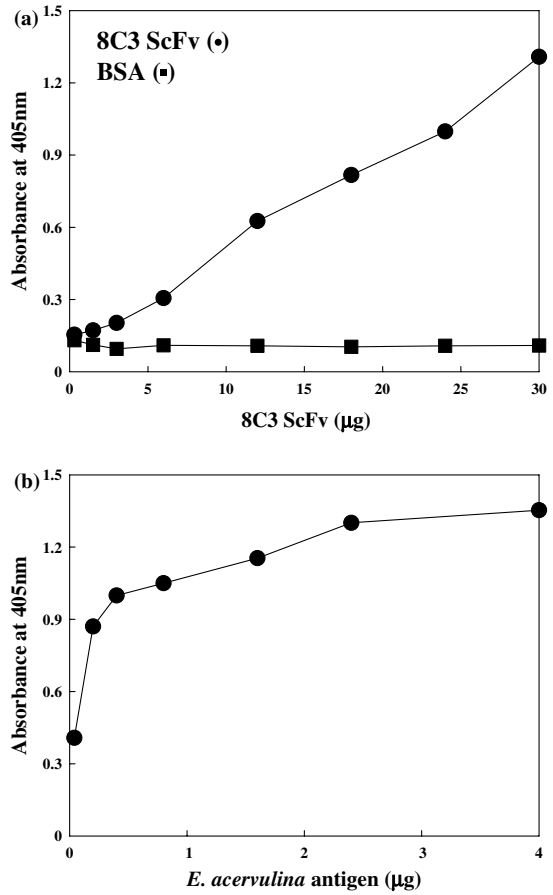

Fig. 4. ELISA of 8C3 ScFv antibodies. *Eimeria acervulina* sporozoite antigens were immobilized on to 96-well plates, after which 8C3 ScFv antibodies were added to each of the wells. BSA was used as a negative control. Binding was detected with horseradish peroxidase-conjugated antibodies and ABTS solution. The plates were read at an absorbance of 405 nm. (a) Varying antibody amounts from 0.3–30 μg or (b) varying antigen amounts from 0.04–4 μg.

construct organized in the reverse (H-L) order (data not shown). This could be due to the inclusion body formation resulting from the positioning of V_H at the NH_2-terminal position of ScFv (Anand *et al.* 1991). The molecular mechanism of generating the antibody diversity in chickens has been shown to involve gene conversion with variable region pseudogenes acting as the sequence donors (Reynaud *et al.* 1987, 1989). Our study may support this notion when we compared the V_L of 8C3 with the pseudogenes (Reynaud *et al.* 1987, Kondo *et al.* 1993). The CDR1, CDR2 and CDR3 of the 8C3 V_L genes were derived from ΨV23, ΨV12 and ΨV13, respectively.

Conclusion

The ability to generate unlimited amount of soluble and functional recombinant ScFv antibodies will facilitate the investigation of their potential therapeutic value in passive immunotherapy against avian coccidiosis.

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